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Economical Biomass production of aerial conidia of *Metarhizium anisopliae* MCC0051 using Agri residues as Solid substrate and its Bioefficacy in controlling *Helicoverpa armigera*

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Abstract: *The Metarhizium anisopliae MCC0051 is a potent, entomopathogenic fungal strain with a wide range of activity against the insect pests. Insect bioassay using of Helicoverpa armigera as a host insect has showed a final mortality above 100% within 8 days. Various agricultural grain brokens were studied to arrive an optimum substrate for Aerial conidia production of Metarhizium anisopliae in Solid Substrate Fermentation. Rice brokens, Wheat brokens, Sorghum brokens, Maize grit and Wheat bran were evaluated. Among all substrates, Rice brokens supported maximum spore production (6.5 x 10^9 conidia/g of substrate).*
Key words: *Agricultural grain wastes, Helicoverpa armigera, Insect assay, Metarhizium anisopliae, Solid substrate fermentation.*

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I. Introduction

All over the world, pests are causing drastic losses in the field of agriculture. In order to prevent this, chemical pesticides and insecticides are widely used which are showing harmful effects on the environment. Agricultural pests and insects are acquiring resistance due to continuous usage of conventional Synthetic pesticides. Due to these dreadful effects, Biological control agents have replaced the chemical pesticides [1].

The most promising alternatives to chemical use are the entomopathogenic fungi such as *Beauveria* and *Metarhizium* which kill a number of pest species [2]. The entomopathogenic fungus *Metarhizium anisopliae* is a Deuteromycete from Monoliaceae family widely distributed in the nature that can be easily found in the soil. This fungus has been studied due to its ability to control insect pests ([3]; [4]; [5]**).** Entomopathogenic fungi selected as Biopesticide should be genetically stable and highly virulent towards the targeted pests [6]. *Metarhizium anisopliae* has proven to be affecting a wide range of insects such as pests, termites and other biological vectors [7].

In the present study, for checking the bioefficacy of *M. anisopliae*, the targeted host selected was *Helicoverpa armigera*. *H.armigera* is one of the pests in Cotton, Chick pea and other crops which are causing great loss all over the world. Due to extensive use of chemical pesticides it has gained resistance to a wide range of conventional pesticides [8].

Metarhizium anisopliae has proven to be controlling the *H.armigera* by secreting the extracellular cuticle degrading enzymes *viz.,* Chitinases, Chitin deacetylase, Chitosanase, Protease, Lipase and secondary metabolites [6] [8]**.**

For large scale production of Entomopathogenic fungi, two methods are mostly used *i.e.* Submerged and Solid state fermentation [9]**.** Solid state fermentation is a natural fermentation process and cost effective method in which agricultural/industrial by products are used as nutrient substrates. In Submerged fermentation maximum Blastospores are formed and these are hydrophilic and have short life span where as in Solid state fermentation aerial conidia are formed. Aerial conidia formed in Solid state fermentation are stable, healthy and lipophilic. As the insect cuticle contains the lipids and waxy material it will be easier for aerial conidia to adhere to the cuticle surface as it has lipophilic nature [10]**.** In the current work, biphasic technology was used in which fungal inoculum is grown in liquid broth and for Aerial conidial formation it is transferred to Solid substrate [11]**.** The present study was undertaken to isolate Potent Entomopathogenic *Metarhizium anisopliae*, *In – vitro* bioassay and to evaluate the substrate for high density aerial conidia production by using different grain by-products *viz.,* Rice brokens, Wheat brokens, Sorghum brokens, Maize grit and Wheat bran [12]**.**

II. Material and methods

2.1. Isolation and maintenance of cultures

For isolation of *Metarhizium anisopliae*, 40 dead cadaver samples of *Helicoverpa armigera* were collected from various agricultural fields of Rejinthal village, Sangareddy District, Telangana. The dead cadavers of *Helicoverpa armigera* were surface sterilized with 1% Mercuric chloride for 30 seconds and washed with distilled water. After surface sterilization, the dead cadavers were chopped into small pieces aseptically [12]**.**

The samples were inoculated on Potato Dextrose agar plates to which 0.01% Chloramphenicol was added and incubated for a week at 28^0C**.** Out of several different fungal species found on PDA plates, 10 *Metarhizium spp.* were isolated based on phenotypic studies and labeled as RSMA0001 to RSMA 0010. These isolated *Metarhizium spp.* cultures were subcultured on PDA slants and stored at 4^0C.

2.2. Preparation of seed inoculums

Metarhizium spp. cultures (RSMA0001 to RSMA 0010) were grown on Potato Dextrose Agar plates at 28^0C for a week. 10 ml of sterile distilled water containing 0.02% of Tween – 80 was added to each Petri plate. Aerial conidia were collected by using Cell scraper into a sterile 10 ml test tube and vortexed for 10 min and filtered. The conidial suspensions were diluted to a concentration of $1.0X10^8$ spores/ml by using sterile distilled water [11]**.**

2.3. Insect Bioassay

For Insect Bioassay studies, 330 no's of in house reared 2nd instar larvae of *H. armigera,* of same size and age were taken. Sunflower leaves were sprayed with *Metarhizium spp.* conidial suspension (RSMA0001 to RSMA 0010) which was adjusted to $1X10^8$ spores/ml using Improved Neubauer chamber method and the leaves were transferred to sterile Petri plates. For control the leaves were sprayed with sterilized water. Three replicates were maintained with 10 *Helicoverpa armigera* larvae per each replication and incubated at 27±1°C and observations were carried out from Day 4 to Day 11. Dead cadavers of *H.armigera* were observed for mycelia development and sporulation. Mortality percentage was calculated and the best *Metarhizium sp.* was chosen for further studies.

2.4. Studies on morphological characteristics of *Metarhizium sp.* by scanning electron microscope

RSMA0004 culture was grown on PDA plates for 5 days at 28^0C and the culture was placed on specimen stubs with double sided adhesive tape of Carbon or Copper. Samples were coated with Gold - Palladium in a JFC- 1600 autofine coater in order to increase the conductivity of the specimen. Samples were then scanned by the JEOL JSM-5600 SEM operated at 5-10 KV. Pictures were taken at appropriate magnification and printed in the required size. At least 20 measurements were made on the morphological characters studied under light and SEM microscopy [13]**.**

2.5. Molecular identification of *Metarhizium sp.* RSMA0004

RSMA0004 was identified till species level by molecular identification studies.

2.6. Evaluation of solid substrates for mass production of *M. anisopliae* MCC0051

Current study was done to evaluate different solid substrates for the production of *M.anisopliae* conidia. 150 g of different solid substrates were pretreated by overnight soaking in water and taken in Autoclavable Polypropelene bags and appropriate moisture content was maintained. Substrate bags were autoclaved at 121^0C for 30 min. After cooling the substrate was inoculated with 1ml of conidia suspension containing $1.0X10^8$ spores/ml and incubated at 28^0C for 7 days [11]**.**

Aerial conidia were counted by taking 1 g of substrate in 10 ml of sterile distilled water containing 0.02% Tween 80. In order to get homogenous suspension of conidia the flasks were kept on shaker for 15 min and filtered. Conidial spore suspension was serially diluted and spore count was assessed by using Standard protocol [14].

III. Results and discussion

3.1. Isolation of *Metarhizium spp.*

Among 40 dead cadavers collected, 10 *Metarhizium spp.* (RSMA0001-RSMA0010) were isolated based on the phenotypic identification.

3.2. Insect Bioassay

Insect Bioassay carried out as per the recommended dose of $1.0X10^8$ conidia/ml on in-house reared 2nd instar larvae of *Helicoverpa armigera*. Among all the *Metarhizium spp.*, RSMA0004 had shown 100% mortality

within 8 days of observation (Table.01). Mycelia growth and sporulation of RSMA0004 was observed on dead cadaver of *H. armigera.* Fig 1.0

Table 01.Mortality of *H. armigera* infested with RSMA0004

Strains	Days after treatment and percent mortality				
	4	5	6	7	8
RSMA0004	61±5.9a	72±1.0a	90.0±5.5a	98.0±2.0a	100.0±0.0a
Control	6.1±1.8c	13.3 ±1.0c	18±3.7c	26.0±5.1c	32.0±3.7c

Values in the same column followed by same letter are not significantly different at 5 % level

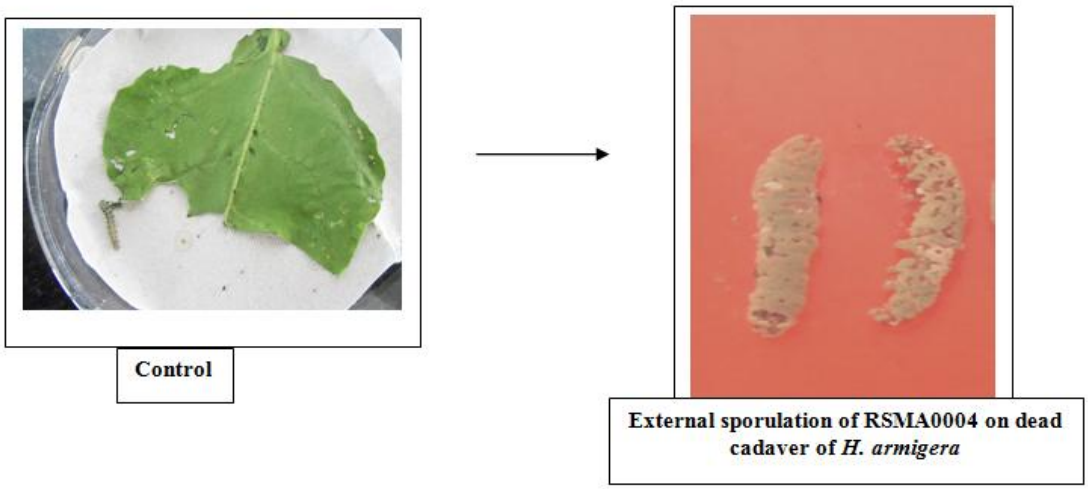

fig 1.0 Morphological variations and external sporulation in 2nd instar larvae of *H.armigera* treated with RSMA0004.

The potent Entomopathogenic fungal strain RSMA0004 has caused 100% mortality on 2nd instar larvae of *H.armigera.* Results shown that the strain had the capability of broad spectrum activity in Biological Insect control as it has the ability to produce Cuticle degrading enzymes like Chitinase and Protease, Formation of appressoria, Hydrophobicity of aerial conidia [6]**.** Fig 1.0

3.3. Studies on morphological characteristics of *Metarhizium sp.* by Scanning Electron Microscope

Morphological studies of RSMA0004 were done by using JEOL JSM-5600 SEM. Photographs were taken at 1000X and 1600X showing the morphology and formation of conidia. The observed morphology was similar to *Metarhizium anisopliae* (Fig 2(a) and Fig 2(b))

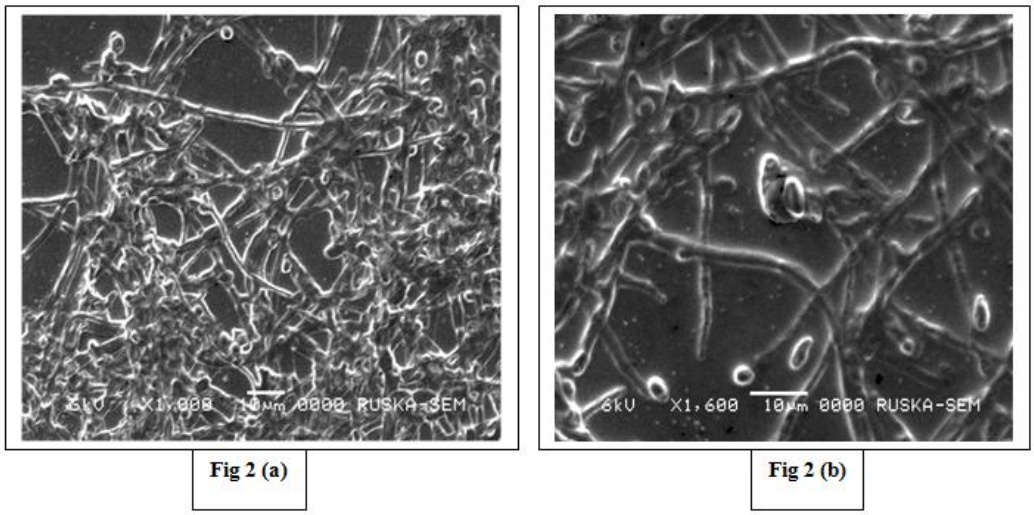

fig 2.0 Morphological characteristics of *M. anisopliae*

3.4. Molecular identification of *Metarhizium anisopliae*

18S rRNA sequencing studies identified and confirmed the RSMA0004 as *Metarhizium anisopliae*. 18S rRNA sequence was checked in BLAST which is a database of NCBI Genbank and identified as *Metarhizium anisopliae*. Maximum identity score of first ten sequences were collected and aligned by using Clustal W. Maximum Likelihood Phylogenetic tree was constructed by using MEGA 7 software. (Fig.3.0)

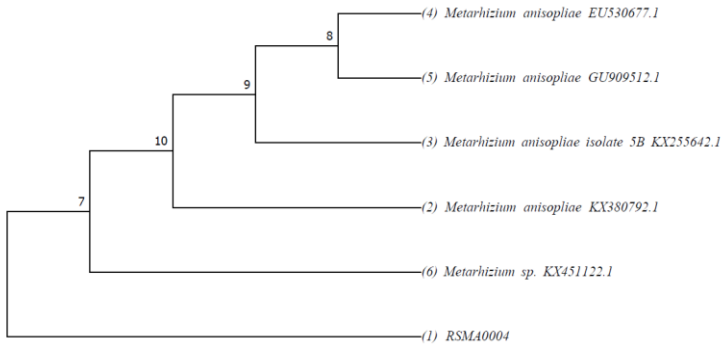

fig.3. Maximum Likelihood tree showing relatedness of RSMA0004 with the *Metarhizium anisopliae*

The identified *M.anisopliae* strain was deposited in Microbial Culture Collection, Pune, India and the accession number was given as MCC0051.

3.5. Evaluation of solid substrates for mass production of *M. anisopliae* MCC0051

Different solid substrates were used for evaluating the best substrate for the Aerial conidial mass production of *Metarhizium anisopliae* MCC0051. Among all the substrates Rice brokens showed high conidial spore count ($6.5X10^9$ cfu/g) followed by Sorghum brokens (3.5×10^9 cfu/g), wheat brokens ($7.5x\ 10^8$ cfu/g), wheat bran ($8.0x\ 10^8$ cfu/g) and Maize grit (1.0×10^9 cfu/g). Fig 4.0

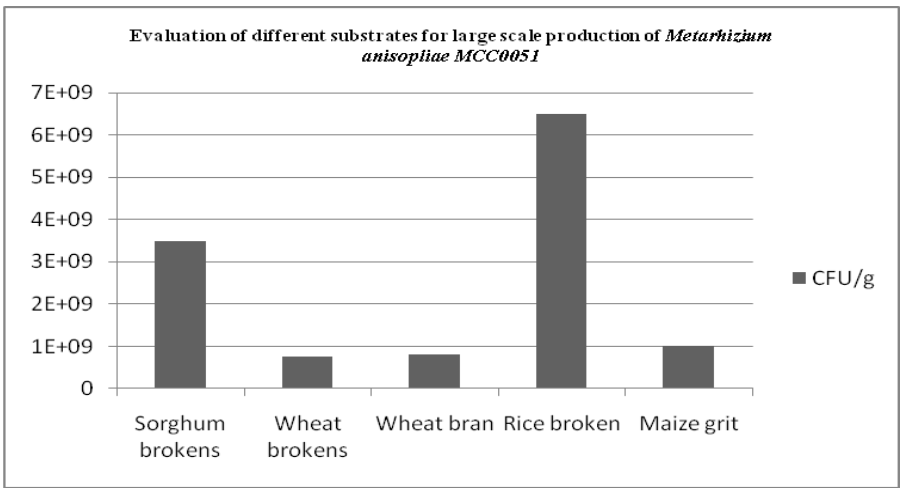

fig 4: Evaluation of different substrates for growing *Metarhizium anisopliae* MCC0051

Among all the substrates tested for high aerial conidia production, Rice brokens showed highest yield as it contains the starch and amylase which on hydrolysis yields glucose and maltose [15]**.** The starch degrading enzymes produced by the fungi releases the maltose which increases the conidial spore production [16]**.**

IV. Conclusion

In present study the potent Entomopathogenic fungi *Metarhizium anisopliae* MCC0051 was isolated from the dead cadavers of *Helicoverpa armigera*. Insect bioassay showed 100 % mortality has been observed on the 2nd instar larvae of *H.armigera*. For the production Aerial condial biomass production, Solid substrate fermentation has been chosen. Among all the substrates, Rice brokens showed high conidial spore count ($6.5X10^9$ spores/g of substrate). It has been concluded that the *Metarhizium anisopliae* MCC0051 can effectively control *Helicoverpa armigera* and can be produced economically employing Rice brokens as the substrate in Solid state fermentation.

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